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(57) Abstract

New human monoclonal antibodies with neutralizing activity against the cytomegalovirus (CMV). A method for preparation of such antibodies is also described, whereby the antibodies are produced from human peripheral blood lymphocytes. The antibodies are specific for epitopes between aa 552-635 of gp58 or aa 67-86 of gp116 and recognize native gp58/gp116 as well.

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HUMAN MONOCLONAL ANTIBODIES AGAINST CYTOMEGALOVIRUS AND METHOD FOR PREPARATION THEREOF.

The present invention concerns new human monoclonal antibodies against cytomegalovirus, and a method for preparation of said antibodies.

Background Art.

Cytomegalovirus (CMV) is a member of the herpesvirus family and is commonly found in the human population, where many individuals are carriers without expressing clinical symptoms of disease. This virus is, however, an important human pathogen with ability to cause disease in immunocompromized hosts, such as transplantation, AIDS and cancerpatients. Furthermore, infants born with congenital infection are at risk of morbidity and long-term sequelae. Consequently, a major interest in the prevention and treatment of clinical disease has developed.

Passive immunization, i.e. the use of preformed virus-specific antibodies have met with great success in e.g. preventing infection of individuals with hepatitis A. In the case of CMV infections, attempts to use polyclonal immunoglobulin preparations containing anti-CMV antibodies, collected from normal, but sero-positive, blood donors, have had some limited success (Meyers et al. Ann. Intern. Med. 98:422, 1983; Winston et al. Ann. Intern. Med. 106:12, 1987). The reasons for only limited success are several; 1.) only a fraction (<0.1%) of the total immunoglobulins are specific for CMV; 2.) immunoglobulins in a polyclonal immunserum also contains specificities that prevents neutralization and are, thus,

detrimental in the battle of the pathogenic virus (Lussenhop et al. Virology 164:362, 1988; Utz et al. J. Virol. 63:1995, 1989); 3.) affinity of the specific immunoglobulins in a polyclonal antiserum is not optimized but is rather a mixture of high and low affinity antibodies, where only a small fraction of these affinities are beneficial for the host receiving the passive immunotherapy. The use of human neutralizing monoclonal antibodies against defined amino acid sequences, known to be involved in protection and not in prevention of virus neutralization, will present a solution to the problems associated with the use of human immune serum, i.e. a polyclonal serum fraction.

The advent of monoclonal antibodies also promted a number of investigators to produce mouse monoclonal antibodies against neutralizing epitopes of CMV. However, mouse monoclonal antibodies are directly disqualified for use in human therapy since these proteins are recognized by the human immune system as being foreign, and are consequently eliminated after a very short period of time, resulting in low or no clinical efficacy; mouse monoclonal antibodies are therefore not further described here.

This leaves human monoclonal antibodies as the only alternative based on use of antibodies for human therapy of the cytomegalovirus. Antigenic targets have been found in surface glycoprotein complexes gp58/116 (gB or gC-1), which have been shown to contain the major neutralizing epitopes (Britt et al. J. Virol. 64:1079, 1990), gp 47-52 (gC-II) and gp 86 (gH or gC-III). gB complex is known to be synthesised as a 130 kDa precursor which is cleaved into two covalently linked molecules, named gp58 and pg116. The N-terminal fragment (gp116) contains one linear, isolate specific epitope and another linear isolate cross-reactive, neutralizing epitope which does not require complement for antibody-mediated biological activity (Meyer et al. J. Gen. Virol. 71:2443, 1990). The gp58 molecule is also known to carry one

immunodominant, neutralizing domain (Kari et al. J. Gen. Virol. 72:1975, 1991; Kneiss et al. J. Virol. 65:138, 1991).

Prior art:

Several human monoclonal antibodies have been described that recognize some of the glycoproteincomplexes (Masuho et al. J. gen. Virol. 68:1457, 1987; Emanuel et al. Clinical applications of monoclonal antibodies, Plenum press, pp 139-148, 1988; Ehrich and Östberg, Therapeutic monoclonal antibodies, Stockton Press, pp. 209-222, 1990; Foung et al. ibid, pp. 173-185; Tomiyama et al. J. Immunol. Methods 131:249, 1990). These antibodies have been characterized to different extent; none of these have been epitope mapped and for some of these antibody-epitopes it is not known on what protein they reside.

From US 5,043,281 (Masuho et al.) there are known human monoclonal antibodies against CMV proteins having molecular weight of approximately 130,000 and 55,000 Da, respectively. These antibodies were prepared by in vitro stimulation of human splenocytes, in the presence of B cell growth factors (BCGF), followed by fusion with mouse myeloma cells, selecting hybridomas which are reactive against CMV and culturing these and obtaining human monoclonal antibodies therefrom.

Definition of Invention.

It has now as a result of over ten years research on human monoclonal antibodies been obtained new, more specific and thus more efficient human monoclonal antibodies against CMV. These human monoclonal antibodies of the present invention are characterized by being reactive against very defined areas (amino acid sequences) of the neutralizing epitopes of gp116 and gp58.

The human monoclonal antibodies against human CMV, of this invention are characterized by

a) being produced from human peripheral blood lymphocytes;
b) recognizing CMV glycoproteins of molecular weight 58 and/or 116 kDa;
c) being specific for epitopes between amino acid nos 552-635 of gp58 and nos 67-86 of gp116, but recognizing the native gp58/gp116 as well;
d) do not have a major reactivity with carbohydrate epitopes;
e) being capable of neutralizing human cytomegalovirus.

The monoclonal antibodies of this invention have been prepared by a process comprising the following steps:

- treating peripheral blood lymphocytes (PBL) from the blood of a CMV seropositive person with a lysosomotopic agent

- infecting the thus treated human lymphocytes with Epstein Barr virus (EBV) to produce human lymphoblastoid cell lines (LCL)

- selecting LCLs producing the monoclonal antibodies of the correct specificity through screening with recombinant proteins encompassing the neutralizing sequences of the 116 and 58 kD glycoproteins

In order to further improve or increase antibody productivity, hybridomas may be established by fusing the antigen-specific LCL to human x mouse heteromyelomas, for instance by using electrofusion technology as described in Ohlin & Borrebaeck, 1992.

Best mode of carrying out the invention:

Human monoclonal antibodies are normally produced from lymphocytes isolated from human spleen, lymphnode, tonsils, or

bone marrow. Peripheral blood mononuclear cells (PBMNC) have always been considered a very difficult cell to use for the production of human monoclonal antibodies since the frequence of antigen-specific B cells is very low in peripheral blood. According to the present invention it is, however, particularly preferred to use PBMNC, since the efficiency in the described approach is significantly higher than any previously described, thus allowing PBMNC to be utilized as a preferred source of immune lymphocytes. Thus, despite the fact that peripheral blood lymphocytes are used, there is no need to in vitro stimulate the PBMNC with antigen prior to immortalization due to the use of a pretreatment with a lysosomotopic agent such as L-leucyl-L-leucine methyl ester, which allows the direct production of human monoclonal antibodies. This is in contrast to e.g. US 5,043,281 (Masuho et al.) which describes a laborious method of in vitro antigen sensitivization for obtaining any human monoclonal antibodies against CMV even if the optimal spleen cells were used, since "there is very little possibility of obtaining the desired hybridomas" unless that approach was used.

A number of different fusion partners are described in the litterature, although heteromyelomas are used with the highest efficiency, and in the present invention the K6H6/B5 or CB-F7 heteromyeloma are preferentially utilized. Furthermore, in the examples of this invention the AD169 strain of CMV is initially used in the neutralization test for characterization of the human monoclonal antibodies, according to the invention.

Human monoclonal antibodies against CMV thus obtained have the following characteristic properties: The human monoclonal antibodies obtained by this invention reacts commonly with many different strains of cytomegalovirus and with many different clinical isolates, but not with other viruses within the herpes family. They react with infected but not uninfected cells and the epitope specificity will be defined by the amino

acid nos. 552-635 (gp58) and nos 67-86 (gp116). The human monoclonal antibodies thus produced are clearly specific for CMV and are for the first time easily derived from peripheral blood lymphocytes.

Embodiments

The following three monoclonal antibodies, having a strong neutralizing activity of CMV, are specifically claimed:

1) ITC88 characterized by being reactive against the amino acid sequence 67-86 of CMV gp116;

2) ITC63b characterized by being reactive with the conformational epitope encompassing amino acid sequences 570-579 and 606-619 of CMV gp58;

3) ITC52 characterized by being reactive with the minimal conformational epitope consisting of sequences 570-579 and 606-619 of CMV gp58;

The amino acid sequences of ITC88, ITC63b and ITC52 are being determined.

All antibodies reacting with the mature, processed gp 58 recognized a conformational epitope, encompassing the sequence between the residues 549 and 635. The minimal common sequence required by all antibodies having this specificity was either amino acid residues 570-579 or 606-619.

According to the method of the invention human monoclonal antibodies with a high neutralizing activity against CMV may be prepared without the need of in vitro antigen stimulation of the lymphocytes, by using using human peripheral blood lymphocytes, which are treated with a lysosomotropic agent such as L-leucyl-L-leucine methyl ester, EBV stimulated and fused with a mouse x human heteromyeloma (K6H6/B5) or CB-F7.

The lysosomotropic agents usable may be any amino acid or peptide ester such as Leu-OMe, Leu-Leu-OMe or Gly-OMe removing cell populations having a negative influence on a method for achieving antigen-specific human monoclonal antibodies.

The monoclonal antibodies according to this invention are more specific than the ones according to the prior art. This is mainly due to the method of selection. By using recombinant proteins encompassing the neutralizing epitopes, only the human monoclonal antibodies having the correct specificity are collected. This assures also the highest natural reactivity achievable for the human monoclonal antibodies.

The method according to the invention makes possible a very efficient preparation of these human monoclonal antibodies. Due to a surprisingly efficient transfection with the EBV it is possible, - in opposition to the teachings of the prior art - to prepare monoclonal antibodies from PBL of seropositive donors. The transfection with the EBV gives a very efficient proliferation of the lymphocytes and gives a high enough population of lymphocytes producing antibodies of the right specificity to make the screening with recombinant proteins possible.

Description of the Figure and Table 1.

The figure shows the neutralizing activity of the monoclonal antibodies of the invention.

Table 1 shows a summary of the reactivities of the antibodies of the invention.

The invention is described in detail by the following examples:

EXAMPLE 1

Production of a neutralizing human monoclonal antibody against gp58 of human CMV (ITC52 and ITC63b)

1. Human peripheral blood mononuclear cells (PBMNC) isolated from normal CMV-seropositive blood donors, are treated with freshly prepared 0.25 mM L-leucyl-L-leucine methyl ester in RPMI-1640, containing 2% calf serum, for 15 minutes at room temperature. The cells were then washed three times with culture medium, containing 2% fetal calf serum.
2. The treated PBMNC are infected with Epstein Barr virus (which is a B lymphotropic virus infecting a high frequency of human B cells) overnight at 37°C, 1 ml of EBV-containing supernatant from the marmoset cell line B95-8 per 10^7 lymphocytes. After infection the cells are washed twice in culture medium and seeded at 3×10^4 cells/well in 96-well microtiter plates with feeder cells (2×10^5 irradiated (3000 rads) PBMNC per well).
3. Lymphoblastoid cell lines (LCL) producing specific antibody and originating from the EBV-infected PBMNC were identified using an antigen-specific (pMbg58; recombinant gB (amino acid # 484-650)) ELISA. Selected LCL were expanded for subsequent somatic cell fusion with the mouse x human heteromyeloma K6H6/B5 or CB-F7.
4. EBV infected cells and the heteromyeloma were fused at a ratio of 1:2, using electrofusion. Cells were seeded at 1.5×10^4 cells/well in 96-well plates in supplemented medium. HAT and 1 μM ouabain were added one day later for hybrid selection.
5. Clones were tested after 1-3 weeks using the antigen-specific (pMbg58; recombinant gB (amino acid # 484-650).
6. Virus neutralization of the produced human monoclonal antibodies: Antibody neutralization of CMV AD 169 was performed using a microneutralization assay on human lung fibroblasts (Gilljam and Wahren, J. Virol. Methods 25:139, 1989).

EXAMPLE 2

Production of a neutralizing human monoclonal antibody against gp116 of human CMV (ITC88)

1. Human peripheral blood mononuclear cells (PBMNC) isolated from normal CMV-seropositive blood donors, are treated with freshly prepared 0.25 mM L-leucyl-L-leucine methyl ester in RPMI-1640 containing 2% calf serum for 15 minutes at room temperature. The cells were the washed three times with culture medium, containing 2% calf serum.

2. The treated PBMNC are infected with Epstein Barr virus (which is a B lymphotropic virus infecting a high frequency of human B cells) overnight at 37°C, 1 ml of EBV-containing supernatant from the marmoset cell line B95-8 per 10^7 lymphocytes. After infection the cells are washed twice in culture medium and grown in bulk culture with feeder cells (2 x 10^5 irradiated (3000 rads) PBMNC/ml) or grown in bulk cultures.

3. Lymphoblastoid cell lines (LCL) producing specific antibodies and originating from the EBV-infected PBMNC were identified using an antigen-specific ELISA. Selected LCL were expanded for subsequent somatic cell fusion with the mouse x human heteromyeloma K6H6/B5 or CB-F7.

4. EBV infected cells and the heteromyeloma were fused at a ratio of 1:1, using polyetylaneglycol medicated fusion. Cells were seeded at 5 x 10^4 cells/well in 96-well plates in supplemented medium, containing HAT and 1 μM ouabain, for hybrid selection. Alternatively, LCL grown in bulk culture was fused with a heteromyeloma at a ratio 1:1, using polyethyleneglycol mediated fusion. Cells were seeded at 5 x 10^4 cells/well in 96 well plates in supplemented medium containing HAT and 1 μM ouabain.

5. Clones were tested after 1-3 weeks using antigen-specific (pHM90-5 amino acid # 28-101)) ELISA.

6. Virus neutralization of the produced human monoclonal antibodies: Antibody neutralization of CMV AD169 was performed using a microneutralization assay on human lung fibroblasts (Gilljam and Wahren, J. Virol. Methods 25:139, 1989).

Reactivity of gp58-specific human monoclonal antibodies with recombinant fragments of gp58 after SDS-polyacrylamide gel electrophoresis and Western blot (+=reactivity; +/-=very weak reactivity; -=no reactivity).

Protein	Sequence	Reactivity	
		ITC52	ITC63B
pMbg58	484-650	+	+
58-Nci	496-621	-	-
gig58-8	484-616	-	-
58-Hin	593-644	-	-
58-Dde	607-638	-	-
gig58-7	484-588	-	-
gig58-2	549-645	+	+
Exo58-315	549-635	+	+
Exo58-36	549-628	-	+/-
Exo58-35	549-628	-	-
Exo58-5A15	552-635	+	+
Exo58-5E39	563-635	-	-

Reactivity of gB-specific human monoclonal antibodies in ELISA to a recombinant fragment (HM90-5) and synthetic peptides which expresses sequences derived from CMV gB (nd=not determined; +=reactivity; -=no reactivity)

Protein or peptide	Sequence	Reactivity		
		ITC52	ITC63B	ITC88
	550-569	-	-	nd
	560-579	+	+	nd
	570-589	+	+	nd
	580-599	-	-	nd
	590-609	-	-	nd
	600-619	+	+	nd
	606-625	+	+	nd
	616-635	-	-	nd
HM90-5	28-101	-	-	+
T7-13	67-86	nd	nd	+

Claims

1. Human monoclonal antibodies against human CMV, characterized by
a) being produced from human peripheral blood lymphocytes;
b) recognizing CMV glycoproteins of molecular weight 58 and/or 116 kDa;
c) being specific for epitopes between amino acid nos 552-635 of gp58 and nos 67-86 of gp116, but recognizing the native gp58/gp116 as well;
d) do not have a major reactivity with carbohydrate epitopes;
e) being capable of neutralizing human cytomegalovirus.

2. Human monoclonal antibody according to claim 1 being ITC88 characterized by being reactive with the amino acid sequence 67-86 of gp116.

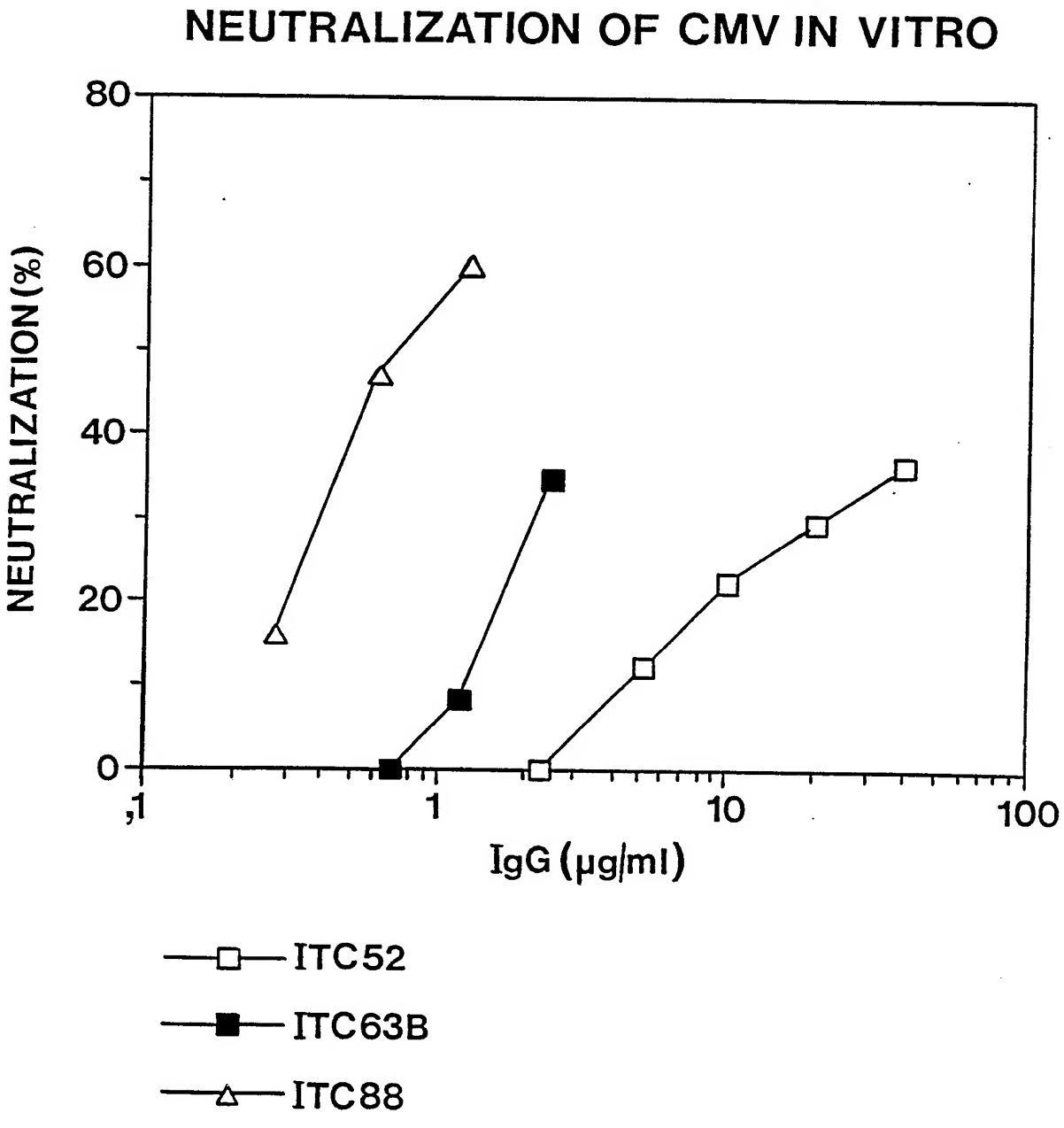
3. Human monoclonal antibody according to claim 1 being ITC52, characterized by being reactive with the minimal conformational epitope consisting of amino acid sequences 570-579 and 606-619 of gp58.

4. Human monoclonal antibody according to claim 1 being ITC63b, characterized by being reactive with the minimal conformational epitope consisting of amino acid sequences 570-579 and 606-619 of gp58.

5. Process for the preparation of human monoclonal antibodies according to claims 1-4 comprising the steps of:
a) treating peripheral blood lymphocytes (PBL) from a CMV seropositive person with a lysosomotopic agent
b) infecting the thus treated human lymphocytes with Epstein Barr virus (EBV) to produce human lymphoblastoid cell lines (LCL)

c) selecting LCLs producing the monoclonal antibodies of the correct specificity through screening with recombinant proteins encompassing the neutralizing sequences of the 116 and 58 kD glycoproteins.

6. Process according to claim 5
characterized by
a) treating human peripheral blood lymphocytes with the lysosomotropic agent L-leucyl-L-leucine methyl ester;
b) EBV infecting the thus treated lymphocytes
c) fusing said infected lymphocytes with a mouse x human heteromyeloma
d) selecting hybridomas producing specific human monoclonal antibodies against the domaines 552-635 of CMV gp58 and 67-86 of CMV gp116 through screening with recombinant proteins
e) culturing said selected hybridomas or a cell line arising therefrom
and
f) obtaining human monoclonal antibodies from said hybridoma or cell lines exhibiting virus neutralizing activity.


NEUTRALIZATION OF CMV IN VITRO
NEUTRALIZATION (%)
80
60
40
20
0
,1
1
10
100
IgG (µg/ml)
ITC52
ITC63B
ITC88

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 93/00343

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: A61K 39/245, C12P 21/08, C07K 15/00, C12N 5/24
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: A61K, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, SCISEARCH, WPIL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of General Virology, Volume 71, 1990, Heidi Meyer et al, "The gp116 of the gp58/116 complex of human cytomegalovirus represents the amino-terminal part of othe precursor molecule and contains a neutralizing epitope" page 2443 - page 2450	1,2
Y	--	1-6
X	Journal of Virology, Volume 65, No 1, January 1991, Norbert Kniess et al, "Distribution of Linear Antigenic Sites on Glycoprotein gp55 of Human Cytomegalovirus"	1,3,4
Y	--	1-6

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl Olof Gustafsson Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 93/00343

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J gen. Virol., Volume 70, 1989, Theresa Banks et al, "A Major Neutralizing Domain Maps within the Carboxyl-terminal Half of the Cleaved Cytomegalovirus B Glycoprotein", page 979 - page 985, see pages 982-984	1,3-6
X	Journal of Immunological Methods, Volume 131, 1990, Takami Tomiyama et al, "Preparation of human monoclonal antibodies against a cytomegalovirus glycoprotein complex of 130 and 55 kDa" page 249 - page 255	2
Y		1-6
T	Journal of General Virology, Volume 73, 1992, H. Meyer et al, "Glycoprotein gp116 of human cytomegalovirus contains epitopes for strain-common and strain-specific antibodies", page 2375 - page 2383, see fig 1 and discussion	2
Y	Clin. exp. Immunol., Volume 84, 1991, M. Ohlin et al, "Characterization of human monoclonal antibodies directed against the pp65-kD matrix antigen of human cytomegalovirus", page 508 - page 514, see page 509	5,6
A	EP, A1, 0248909 (TEIJIN LIMITED), 16 December 1987 (16.12.87)	1-6

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/07/93

International application No.

PCT/SE 93/00343

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0248909	16/12/87	AU-B- 590656	09/11/89
		AU-A- 6721887	30/06/87
		US-A- 5043281	27/08/91
		WO-A- 8703602	18/06/87